

Hyperplasia and fibrosis in mice with conditional loss of the TSC2 tumor suppressor in Müllerian duct mesenchyme-derived myometria

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ABSTRACT: Uterine leiomyomata are the most common tumors found in the female reproductive tract. Despite the high prevalence and associated morbidities of these benign tumors, little is known about the molecular basis of uterine leiomyoma development and progression. Loss of the Tuberous Sclerosis 2 (TSC2) tumor suppressor has been proposed as a mechanism important for the etiology of uterine leiomyomata based on the Eker rat model. However, conflicting evidence showing increased TSC2 expression has been reported in human uterine leiomyomata, suggesting that TSC2 might not be involved in the pathogenesis of this disorder. We have produced mice with conditional deletion of the *Tsc2* gene in the myometria to determine whether loss of TSC2 leads to leiomyoma development in murine uteri. Myometrial hyperplasia and increased collagen deposition was observed in *Tsc2*^{ckO} mice compared with control mice, but no leiomyomata were detected by post-natal week 24. Increased signaling activity of mammalian target of rapamycin complex 1, which is normally repressed by TSC2, was also detected in the myometria of *Tsc2*^{ckO} mice. Treatment of the mutant mice with rapamycin significantly inhibited myometrial expansion, but treatment with the progesterone receptor modulator, mifepristone, did not. The ovaries of the *Tsc2*^{ckO} mice appeared normal, but half the mice were infertile and most of the other half became infertile after a single litter, which was likely due to oviductal blockage. Our study shows that although TSC2 loss alone does not lead to leiomyoma development, it does lead to myometrial hyperplasia and fibrosis.

Key words: uterine fibroid / mTOR / tumorigenesis / infertility / TSC2

Introduction

Uterine leiomyomata (fibroids) are benign tumors and represent the most common neoplasms in reproductive-aged women. The prevalence of this gynecologic disorder is dependent upon several factors including age, estrogen status, race/ethnicity, detection method and diagnostic criteria. Notably, 80% of cases are in asymptomatic women, whereas the number of symptomatic cases is ~2–25% (Baird *et al.*, 2003). The symptoms of leiomyomas may consist of abnormal uterine bleeding, pelvic pressure and pain and reproductive dysfunction (Terry *et al.*, 2010). The most common symptom, menorrhagia (excessive menstrual bleeding with regular intervals), is most often associated with the presence of submucosal leiomyomas. Despite their significant impact on women's health and reproductive medicine, the etiology of uterine

leiomyomas has not been clearly determined, nor has their relationship with the mechanisms associated with excessive bleeding been identified. In the absence of a targeted therapy, there are several hormonal treatment options that act at different levels of the hypothalamic–pituitary–gonadal axis. Traditional hormone mediated treatments have variable effects (Islam *et al.*, 2013) and selective progesterone receptor modulators have garnered increased interest recently (Talauiikar and Manyonda, 2012). However, hysterectomy remains the only definitive treatment option and leiomyomata are the leading indication for hysterectomy in woman of reproductive age annually (Rein, 2000; Walker and Stewart, 2005).

There are numerous indications that the development of leiomyomata is related to hormonal status (Blake, 2007). For example, leiomyomas do not normally occur in prepubertal girls and the incidence of disease

increases until menopause after which the fibroid burden decreases and symptoms resolve. During pregnancy, fibroids will often decrease in size and can become undetectable by post-partum ultrasound examination (Baird and Dunson, 2003; Laughlin et al., 2010). This might be a combined effect of hormonal status during pregnancy and the reorganization of the uterus after delivery. Alternatively, fibroids can become ischemic and necrotic after parturition, providing symptomatic relief to patients (Burbank, 2004). Epidemiologic studies suggest that the risk of developing leiomyomata is lower for multiparous women and for women with a higher age of menarche (Wise et al., 2005; Terry et al., 2010). Leiomyomata are of clonal origin, suggesting their origin from a specific sub-type of cells in the myometrium (Stewart and Morton, 2006; Terry et al., 2010). A variety of cytogenetic abnormalities have been detected in ~40% of the leiomyomas (Rein et al., 1998), that consist of both gain of function and loss of function mutations, suggesting the possibility that different genetic mechanisms could be responsible for one common clinical disease, or that there are multiple mutations affecting one common downstream signaling pathway. Recently a mutation in the transcription regulator mediator complex subunit 12 (MED12) was shown to be mutated in 70% of human uterine leiomyoma (Makinen et al., 2011) but the mechanisms disrupted by the mutated MED12 that lead to leiomyoma development have not been described.

Deregulation of the mammalian target of rapamycin (mTOR/FRAP1) pathway, a master regulator of cellular proliferation, has also been implicated in the emergence of uterine leiomyomata through TSC2 loss (Everitt et al., 1995), by microarray analyses (Crabtree et al., 2009) and by constitutive activation of β -catenin through an as yet unknown mechanism (Tanwar et al., 2009). The mTOR pathway senses and integrates multiple signals related to cell proliferation and survival such as stress, AMP/ATP ratio and growth factors in the cellular microenvironment (Laplante and Sabatini, 2012). Tuberous Sclerosis Complex proteins, TSC1 and TSC2, integrate the signals of multiple pathways to regulate mTOR complex 1 (mTORC1) activation, via the intermediate protein, RAS homolog enriched in brain (RHEB) which regulates its kinase activity. Patients with Tuberous Sclerosis Complex, a human autosomal dominant hereditary disease that is linked to germline mutation in the *TSC1* or *TSC2* genes, develop soft tissue neoplasms. A link between TSC2 loss, activation of the mTORC1, and uterine leiomyomas was established by the Eker rat, which has a deletion of one of the *Tsc2* alleles; ~72% of aged female Eker rats develop microscopic uterine leiomyoma-like lesions (Everitt et al., 1995).

We and others have previously shown that conditional loss of TSC1 in murine gonads and reproductive tracts using Amhr2-Cre results in complete female infertility from a combination of oviductal occlusion and defective embryo implantation that we speculated was due to disrupted progesterone receptor activity (Tanaka et al., 2012; Daikoku et al., 2013). We have also shown that loss of either TSC1 or TSC2 in the endometrial stroma drives endometrial epithelial hyperplasia in older mice (Tanwar et al., 2012). In order to determine whether loss of TSC1 or TSC2 would result in increased incidence of uterine leiomyomata development, we used mice with the Cre recombinase gene knocked into the Anti-Müllerian hormone/Müllerian inhibiting substance type II receptor gene (*Amhr2*) (Jamin et al., 2002) to specifically delete their genes in the endometrial stroma and myometrium (Teixeira et al., 2001; Hernandez et al., 2007; Tanwar et al., 2009, 2011). In this study, we show that although no phenotype is observed in the *Tsc1*^{CKO} myometria, hyperplasia and fibrosis are prevalent in the myometria of *Tsc2*^{CKO} mice. However,

uterine leiomyomas are not observed in mice aged up to 6 months. Additional studies with *Tsc2*^{CKO} mice show that oral rapamycin therapy significantly inhibits, whereas mifepristone implants induce, myometrial hyperplasia.

Materials and Methods

Mouse genetics and husbandry

The mice used in this study were housed under standard housing conditions and used with protocols approved by the Institutional Animal Care and Use Committee of Massachusetts General Hospital. *Amhr2*^{tm3(cre)Bhr} (*Amhr2-Cre*) mice (Jamin et al., 2002) (Kindly provided by Dr. Richard Behringer at MD Anderson, Houston, TX, USA) were bred with *Tsc2*^{tm1.1Mjg} mice (Hernandez et al., 2007) (Kindly provided by Dr. Michael Gambello at the University of Texas, Houston Medical School) to generate *Tsc2*^{CKO} mice. *Tsc1*^{CKO} mice were produced using the same mating scheme with *Tsc1*^{tm1Djk/J} (Kwiatkowski et al., 2002) purchased from Jackson Laboratories (Bar Harbor, ME, USA). Similarly, *Amhr2-Cre* mice were mated with *Ctnnb1*^{tm1Mmt} (Harada et al., 1999) (Kindly provided by Dr. Mokoto Taketo at Kyoto University) to generate *Amhr2*^{tm3(cre)Bhr/+}; *Ctnnb1*^{tm1Mmt/+} mice, hereafter called constitutively activated (CA) β -catenin mice. Mice used in this study were maintained on a C57BL/6;129/SvEv mixed genetic background. The DNA from tail biopsies was used to genotype mice using standard PCR protocols. Comparisons were done between *Tsc2*^{CKO} mice and littermate controls whenever possible. Recombination of the *Tsc2*^{loxP/loxP} allele in the appropriate tissues was confirmed by PCR, including with DNA from laser capture microdissection tissue sections. Fertility was assessed at 6 weeks post-natal and for a further 12 weeks by mating with control male mice. Steroid hormone assays were performed by the Ligand Assay and Analysis Core at the University of Virginia, Charlottesville, VA, USA. Gross photos were taken with either a Nikon D60 camera or a Nikon SMZ1500 microscope equipped with a Spot Camera (Diagnostic Instruments, Sterling Heights, MI, USA).

Drug administration

Female mice ($n = 4$ in each treatment group) at 9 weeks of age were treated with 200 μ l Sirolimus (also known as rapamycin or Rapamune, Wyeth Pharmaceuticals, Philadelphia, PA, USA) or an equivalent volume of vehicle control (The American Lecithin Company, Oxford, CT, USA) three times/week for a period of 6 weeks by oral gavage. Mice ($n = 4$ in each treatment group) at 9 weeks of age were treated with mifepristone in one single subcutaneous 60-day controlled release capsule (9.75 mg) or control capsule implant (Innovative Research of America, Sarasota, FL, USA). The mice were euthanized 1–3 days after the last treatment day for tissue collection and analysis.

Histology, immunofluorescence, immunohistochemistry and analyses

Mice ($n \geq 3$) were euthanized at 6, 12, 18 and 24 weeks for gross, histological and morphometric analyses of the female reproductive tracts. The isolated tissues were fixed in 4% paraformaldehyde at 4°C overnight, then dehydrated in ascending ethanol concentrations and xylene, embedded in paraffin wax and completely sectioned. Tissue slides (6 μ m) were heated, deparaffinized with xylene and rehydrated in descending ethanol concentrations and distilled water. Hematoxylin and eosin staining was performed using standard histological techniques. Masson's Trichrome staining was performed according to the manufacturer's instruction (Sigma, St. Louis, MO, USA). For immunofluorescence (IF) and immunohistochemistry (IHC), antigen retrieval was done by boiling the slides in 0.01 M citrate buffer (pH 6) for

10–20 min. Blocking was done with bovine serum albumin for α SMA or with second antibody serum for TSC2 in PBS for 1 h at room temperature in a dark humidified chamber. The tissue sections were subsequently incubated with primary antibodies at 4°C overnight, washed, then incubated for an hour with secondary antibody at room temperature for TSC2 or with the Envision HRP kit (Dako, Carpinteria, CA, USA) according to the manufacturer's instructions for pS6 and TSC1. The following antibodies were used: α SMA-Cy3 (1:2500) from Sigma; pS6 (1:75), TSC1 (1:1000), and TSC2 (1:100) from Cell Signaling Technologies, Beverly, MA, USA. The nuclei were counterstained with 4',6'-diamidino-2-phenylindole (DAPI) for immunofluorescence or by hematoxylin for immunohistochemistry. Photographs of the sections were made with a Nikon Eclipse Ni microscope equipped with Nikon DS-Qi1Mc and DS-Fi2 digital cameras. Quantitative analyses were done using NIS Elements 4.0 software using a minimum of three replicates of random sections/tissue and $n \geq 3$ mice per genotype or treatment group for myometrial area. For hyperplasia/hypertrophy analyses, whole α SMA- and DAPI-stained uterine sections were imaged at 2–4 \times magnification, while selected areas of myometrium were imaged at $\times 20$ for optimal resolution of thresholding and cell counting. Four myometrial counting areas were selected per duplicate adjacent tissue sections for a total of 24 nuclear counts per mouse myometrial sample with $n = 9$ different mice each for controls and mutants. The total nuclear counts per mouse myometrium were extrapolated from average counts of the four areas as calculated by the NIS Elements software.

Tsc2 mRNA in situ hybridization

Mouse uteri ($n = 6$ controls and mutants) were fixed with 4% paraformaldehyde and paraffin embedded. Sections (6 μ m) were processed according to the RNAscope 2.0 HD instructions, (Advanced Cell Diagnostics, Hayward, CA, USA). Mm_dapB, Mm-UBC (Ubiquitin C), and Mm-TSC2 probes that were specific for the deleted exons of TSC2 were supplied by the manufacturer for the negative control, positive control and test probes, respectively. Both control and mutant tissue sections were evaluated for a positive signal as the development of brown, punctate dots and clusters under brightfield microscopy, using a Nikon Eclipse and NIS Br Elements software. A semi-quantitative scoring method was applied using a scale of 0–4, with 0 indicating background or little staining and 4 indicating widespread staining, as recommended by the manufacturer for each of the tissue sections ($n \approx 4$ fields in $n \approx 3$ sections \times 2 slides for each of 6 uteri). Significant differences between the median stain intensities of control and mutant groups were tested by rank sum analyses.

Statistical analyses

The mean or median values from $n \geq 3$ mice were analyzed for statistically significant differences by GraphPad Prism software (GraphPad Software, La Jolla, CA, USA) using the paired student *t*-test, Mann–Whitney rank sum test, or ANOVA, as appropriate. Significance was assigned at $P \leq 0.05$.

Results

Since we did not observe leiomyoma development in mice with TSC1 loss (Tanaka et al., 2012), we analyzed whether TSC2 loss affected either female fertility or myometrial pathology, with the expectation that uterine leiomyomata would develop in these mice with deletion of both *Tsc2* alleles. Recombination of the floxed allele was confirmed by PCR of DNA isolated from whole ovaries, oviducts, and uteri and in myometria by laser capture microdissection (Supplementary data, Fig. S1A and B). *Tsc2* expression was shown to be diminished to a variable degree by *in situ* hybridization with *Tsc2* mRNA probes (Supplementary data, Fig. S1C) and by immunohistochemistry for TSC2 (Fig. 1A and B).

Tsc2^{ckO} ovaries appeared normal at both 6 and 24 weeks post-natal (Supplementary data, Fig. S2A), except for the ovarian surface epithelial hyperplasia observed in nearly 50% of the ovaries examined (Tanwar et al., 2014), and neither the estradiol nor the progesterone profiles of the mice appeared to be affected by *Tsc2* deletion (Supplementary data, Fig. S2B). Unlike the *Tsc1*^{ckO} female mice, which were always sterile (Tanaka et al., 2012), $\sim 50\%$ of *Tsc2*^{ckO} females were subfertile with smaller litters (Table 1); the other 50% were sterile from the beginning of the analysis. All of the dams had only a single litter, except for one that had two, before becoming sterile, which we attributed to progressive oviductal blockage commonly observed in older *Tsc2*^{ckO} females (Supplementary data, Fig. S2A,g). Since the oviductal phenotype is similar to that which we have reported for the *Tsc1*^{ckO} female mice (Tanaka et al., 2012) and because of the role ascribed to TSC2 in uterine fibroid development in the Eker rat (Everitt et al., 1995), we chose to concentrate our subsequent analyses on the myometrial phenotypes.

Myometrial hyperplasia and fibrosis with loss of TSC2

Examination of *Tsc2*^{ckO} uteri showed remarkable expansion of the myometria compared with controls (Fig. 1C–F), which was confirmed by α -Smooth Muscle Actin (α -SMA) immunofluorescence (Fig. 1G and H). Additionally, much of the expansion observed in the *Tsc2*^{ckO} myometria was composed of extracellular matrix as determined by Masson's trichrome staining (Fig. 1I and J), suggesting increased levels of fibrosis. The TSC2 heterodimer regulates the activity of mTORC1, a master regulator of proliferation (Laplane and Sabatini, 2012), and loss of either TSC1 or 2 should result in deregulated mTORC1 independently of any external signaling mechanisms upstream of TSC2. Increased levels of phospho-S6, a downstream target of mTOR activity, by immunofluorescence (Fig. 1K and L) indicated deregulated activation of mTORC1, suggesting that the hypertrophy is the result of loss of TSC2. Unlike the results we observed in *Tsc2*^{ckO} myometria, histological and immunofluorescence analyses did not show any myometrial phenotype in *Tsc1*^{ckO} mice other than increased extracellular matrix at 40 weeks post-natal (Supplementary data, Fig. S3). Thus, only the *Tsc2*^{ckO} myometria were used in our subsequent analyses.

Unlike CA- β -catenin uteri (Tanwar et al., 2009), no leiomyomata were detected in $n > 80$ *Tsc2*^{ckO} uteri from mice aged 6 weeks up to 6 months post-natal by gross inspection. We assessed whether microscopic leiomyomas were present in $n > 24$ *Tsc2*^{ckO} uteri by using Masson's trichrome staining, which stains collagens in the extracellular matrix (ECM) blue, the excess of which characterizes the signature phenotype of uterine leiomyomata. In Fig. 2A–H, we show that in control myometria, ECM was limited to the thin basement membrane surrounding the longitudinal muscle bundles and to the interface between the longitudinal and circular smooth muscle layers. By 12 weeks and afterwards, faint blue staining was occasionally visible interspersed within the circular muscle layer of controls (Fig. 2B and F). In contrast, stronger blue staining was observed throughout the myometria of *Tsc2*^{ckO} mice at all ages (Fig. 2I–P). By 24 weeks (Fig. 2L and P), the accumulation of ECM in the *Tsc2*^{ckO} myometria, particularly in the antimesometrial pole, was very high as shown by the strong blue staining compared with controls.

The degree of myometrial hypertrophy in the *Tsc2*^{ckO} mice was quantified by IF with α SMA staining followed by analyses of the diameter, area

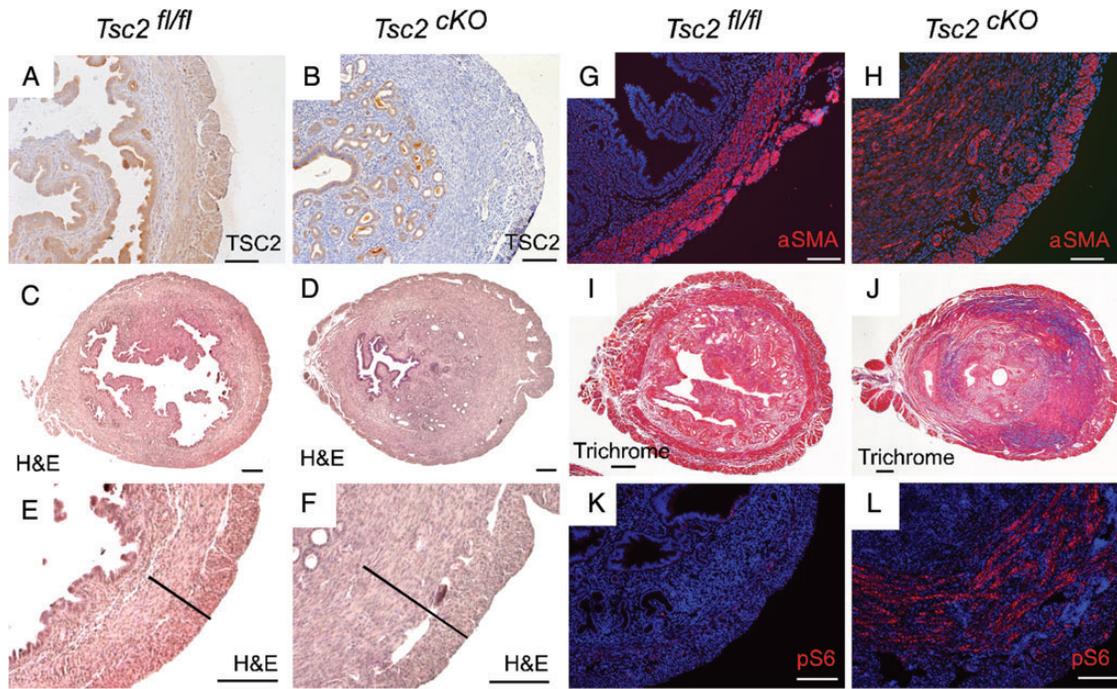


Figure 1 Myometrial expansion and mTORC1 activity are induced by the loss of TSC2. Immunohistochemistry for TSC2 is shown in cross sections of control and *Tsc2^{CKO}* uteri in (A) and (B), respectively. Low power (C and D) and higher power (E and F) magnification of H&E sections of control and mutant uteri. The line in (E) and (F) indicates the width of the myometria in *Tsc2^{CKO}* uteri is expanded compared with the control uteri. Immunofluorescence for α SMA confirms that the expansion observed in the *Tsc2^{CKO}* myometria is composed of smooth muscle (G and H). Trichrome staining of the control and *Tsc2^{CKO}* myometria (I and J, respectively) indicates that extracellular matrix deposition (shown with blue staining) also contributes to myometrial expansion in the mutant uteri. Increased immunofluorescence for phospho-S6 in the mutant myometria (L) compared with control (K) indicates activation of mTORC1. Bars equal 100 μ m. Nuclei are stained with either hematoxylin or DAPI.

Table 1 *Tsc2^{CKO}* mice are subfertile (12 weeks of mating).

	Total litters	Total pups	Pups/litter	Litters/mouse
<i>Tsc2^{loxP/loxP}</i> (n = 7)	16	106	6.6 \pm 0.6	2.7 \pm 0.3
<i>Tsc2^{CKO}</i> (n = 27)				
Fertile (n = 19)	20	78	4.9 \pm 0.6	1.1 \pm 0.1
Infertile (n = 18)	0	0	0	0

and ratio of myometrium area to total uterine area in cross section. In Fig. 3, we show that after post-natal 6 weeks, when females have reached sexual maturity, the myometria of *Tsc2^{CKO}* mice (Fig. 3E–H) have significantly increased diameters and areas compared with controls (Fig. 3A–D) at most time points examined (Fig. 3I and J). At 6 weeks (Fig. 3A, E and K) the ratio of myometrial area to total uterine area was the same, but by 12 weeks (Fig. 3B, F and K), the ratios of myometrial and total uterine area were significantly increased and remained increased thereafter (Fig. 3C–H and K). Additionally, the uteri examined at 12 weeks were all collected and examined from both control and mutant mice in estrus to avoid any confounding effects of estrous cycling. These results confirm that TSC2 loss induces myometrial hypertrophy. Comparison of the number of DAPI-stained nuclei in the *Tsc2^{CKO}*

and control myometria (Fig. 3L) indicated that the myometrial hypertrophy in the mutants was due to hyperplasia of the myometrial cells.

Rapamycin inhibits myometrial hypertrophy

If the myometrial hypertrophy is the result of the deregulated activity of mTORC1, rapamycin (Sirolimus) therapy should mitigate this effect. We observed a significant decrease in myometrial hyperplasia when *Tsc2^{CKO}* mice were treated with Sirolimus by oral gavage for 6 weeks compared with vehicle treatment (Fig. 4A, B and E). The mean ratio of myometrial surface to whole cross section uterus surface was significantly decreased from 0.72 to 0.58. Analysis of pS6, a downstream target of mTORC1 activation, by IHC confirmed inhibition of mTORC1 activity by Sirolimus (Supplementary data, Fig. S4). We have previously shown that mice expressing a gain of function allele of β -catenin in myometria (CA β -catenin) also develop myometrial hyperplasia and, in some cases, uterine leiomyomas (Tanwar et al., 2009). In contrast to *Tsc2^{CKO}* uteri, Sirolimus therapy appeared to increase the myometrial ratio in CA β -catenin mice (Fig. 4C, D and F), which might be at least partially due to the decrease in overall cross sectional area of these uteri. These results indicate that inhibition of mTORC1 activity by Sirolimus could be beneficial for treating patients with dysregulated TSC2.

Inhibition of progesterone receptor (PR) activity has been used in women with symptomatic leiomyomata therapeutically with variable success (Sangkomkamhang et al., 2013). We treated mice for 6 weeks

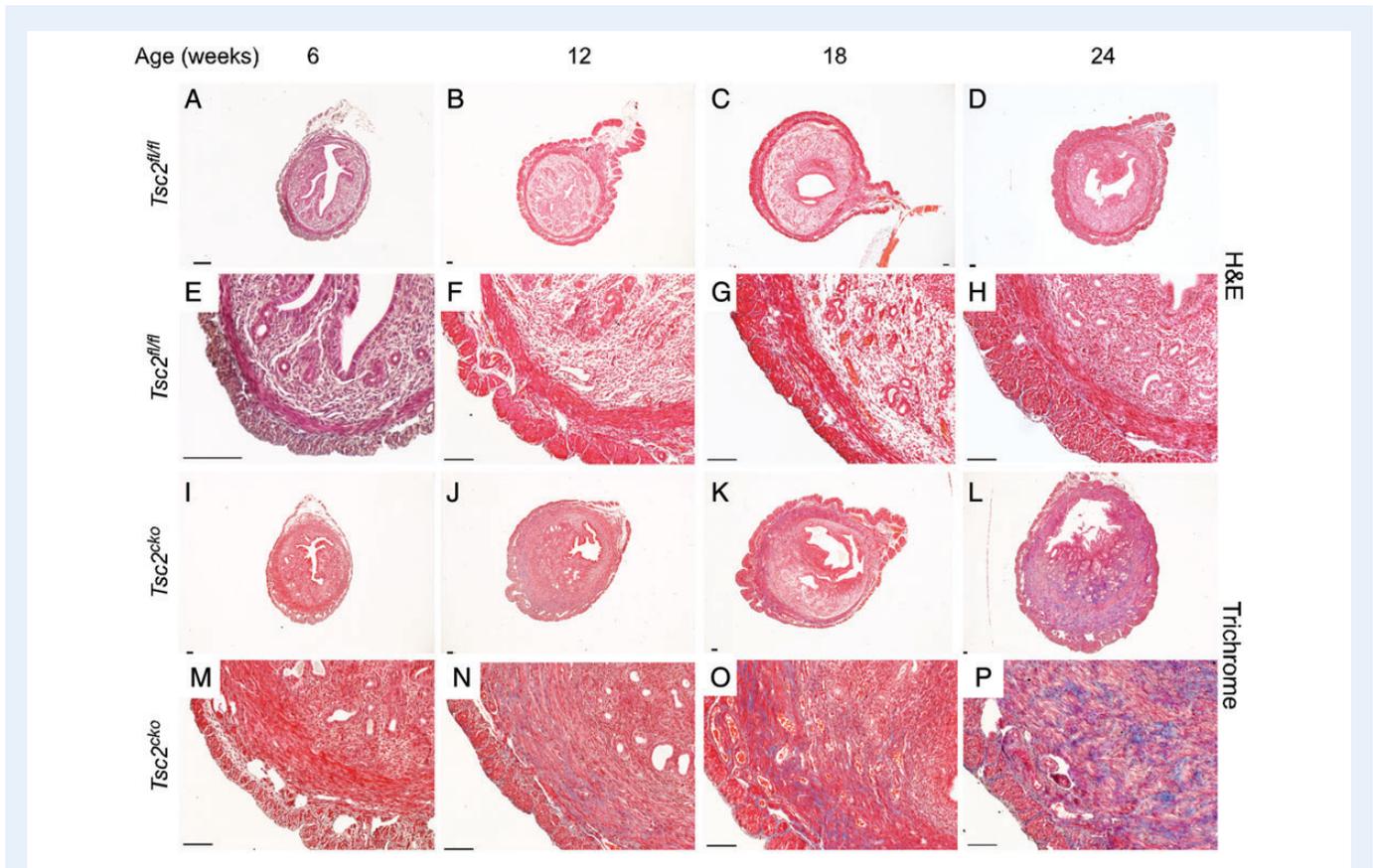


Figure 2 Trichrome staining of $Tsc2^{CKO}$ myometria indicates fibrosis. Low and high magnification photos of typical Masson's trichrome-stained myometria from (representative of four control and mutant uteri at the indicated age) control (**A–H**) and mutant (**I–P**) are shown at the start of sexual maturity at 6 weeks through 24 weeks of age. Very little blue-stained ECM was observed in young uteri from either control or mutant mice (**A, E, I** and **M**). Faint blue staining was observed at 12 weeks in the mutant myometria (**J** and **N**) and became progressively stronger with age (**K, O, L** and **P**). Bars equal 100 μm .

with mifepristone (RU486), a selective progesterone receptor modulator, to determine whether PR inhibition affected myometrial hyperplasia in both $Tsc2^{CKO}$ and CA β -catenin mice. Fig. 5A, B and E shows that mifepristone appeared to induce myometrial hyperplasia in $Tsc2^{CKO}$ mice. Interestingly, mifepristone slightly inhibited myometrial hyperplasia in CA β -catenin mice and appeared to decrease α -SMA expression in the inner smooth muscle layer (Fig. 5C, D and F). Taken together with the results from the Sirolimus-treated mice, it appears that the effect of antiproliferation therapies is dependent upon the underlying mechanism driving the myometrial hyperplasia.

Discussion

We have been exploiting the expression of *Amhr2*-driven Cre in the Müllerian duct mesenchyme-derived female reproductive tract to understand both uterine development and the pathogenesis of uterine disease by manipulating the expression of tumor suppressors and oncogenes. One of the pathways we are most interested in is the PI3K-mTOR pathway, which integrates signaling from various external stimuli through the cell surface receptor tyrosine kinases to regulate mTORC1 activity. In this report we show that conditional deletion of *Tsc2* in murine uteri results in myometrial hyperplasia and fibrosis. In contrast, we did not detect a myometrial phenotype with conditional

deletion of *Tsc1* (Supplementary data, Fig. S3). A bias toward sporadic *TSC2* mutation was observed in early studies of Tuberous Sclerosis (TSC) patients, which was correlated with a significantly higher risk of developing mental retardation (Jones et al., 1997). A subsequent study with a large cohort of unselected TSC patients also showed that those with *TSC1* mutations tended to present with a milder phenotype of the disease in several diagnostic affected tissues, including the brain, retina, kidneys and face (Dabora et al., 2001; Curatolo et al., 2008). Thus, it appears that, as in humans, mutation or deletion of *Tsc2* compared with *Tsc1* is more detrimental in mice at least in the myometria.

The ability of embryos to implant and produce live births in $Tsc2^{CKO}$ (Table I), but not in $Tsc1^{CKO}$ (Tanaka et al., 2012) mice, uteri indicates that loss of either TSC1 or TSC2 in the Müllerian duct-derived endometrial stroma does not phenocopy each other in that uterine compartment, as well. This is in contrast to the myometrium, where TSC2 loss produces a more severe phenotype than does loss of TSC1. The predominant function of TSC1 has been thought to be that of stabilizing TSC2, which is considered the functional portion of the heterodimer because of its GTPase activity. Thus, the lack of implantation failure in the $Tsc2^{CKO}$ uteri, as evidenced by the ability of half the mice to deliver litters, suggests that TSC1 has an additional function in the endometrial stroma independent of TSC2. Additionally, many different proteins

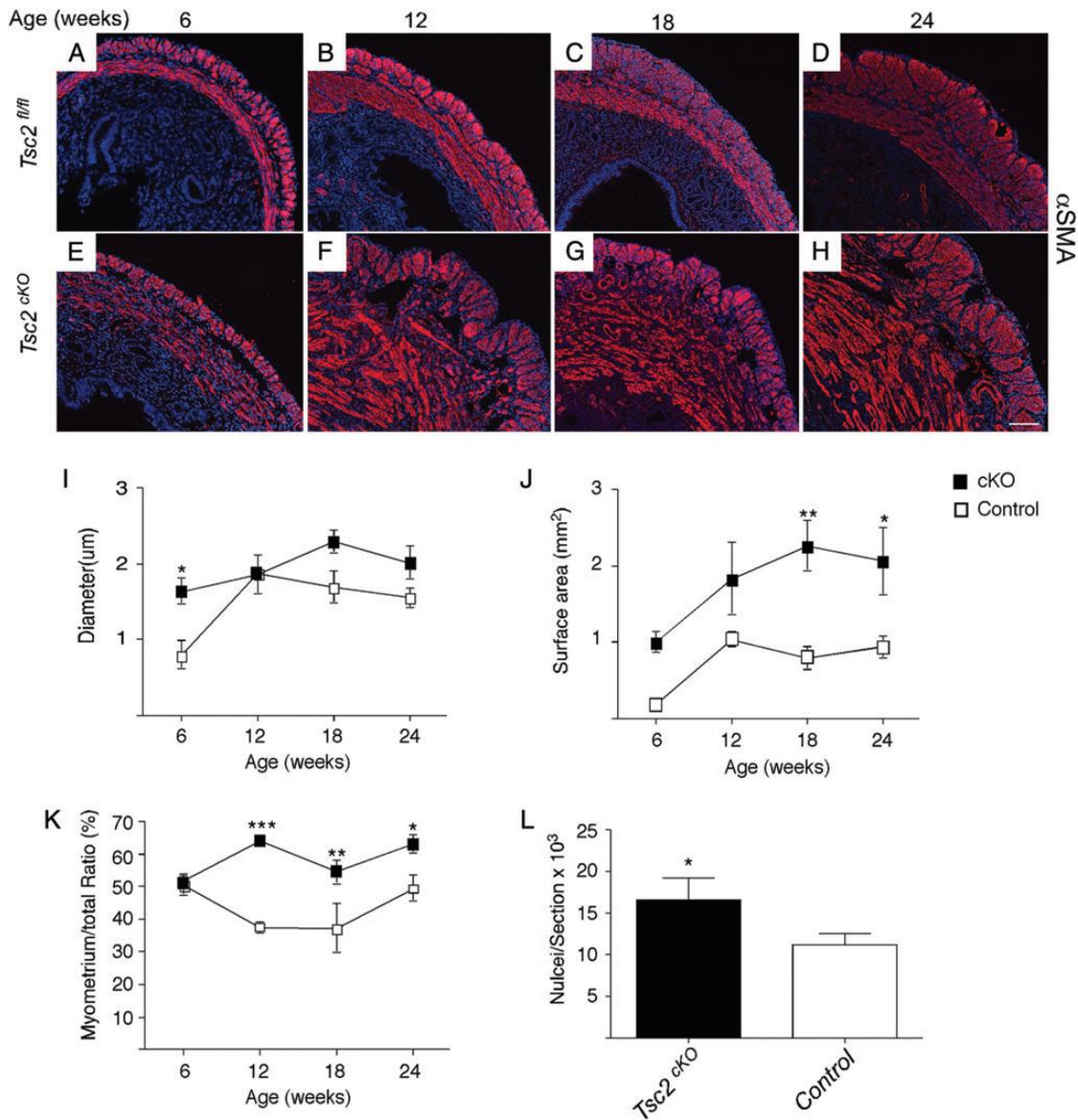


Figure 3 Myometrial hyperplasia in *Tsc2^{CKO}* uteri. Myometrial composition of uterine cross sections from control (A–D) and *Tsc2^{CKO}* (E–H) mice ($n = 4$ each) at the indicated ages was analyzed by α SMA immunofluorescence. Uterine diameters (I) and the α -SMA+ myometrial surface areas (J) were measured at the indicated ages, and the mean \pm SEM were plotted. The ratios of myometrial area to the total uterine area (K) were calculated and the mean \pm SEM plotted. Statistical analysis was performed by two-way ANOVA and Bonferroni post-test. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. Total number of nuclei were counted in the α -SMA+ myometrial areas in cross sections of $n = 9$ different uteri each of control and *Tsc2^{CKO}* mice. The mean \pm SEM are shown plotted in (L). * $P < 0.05$ by student's t -test. Nuclei are stained with DAPI (A–H). Bar = 100 μ m.

involved in signaling pathways other than PI3K have been shown to interact with the TSC1/2 heterodimer (Au et al., 2004). In the case of TSC1 alone, one of the reported interactions is with the ezrin-radixin-moesin (ERM) family of actin-binding proteins (Lamb et al., 2000), which are important regulators of the actin cytoskeletal dynamics by mechanisms involving the regulation of RhoA (Hirao et al., 1996). Overexpression of TSC1 *in vitro* was also shown to induce RhoA activation in that report, suggesting that TSC1 might function in cellular morphology and adhesion, independently of TSC2. There are profound changes to the endometrial stroma cell morphology and function during decidualization, which is a

process required to maintain the early stages of pregnancy (Ramathal et al., 2010; Cha et al., 2012). Perhaps the loss of TSC1 affects the ability of the endometrial stromal cells to decidualize because of the lost interaction with ERMs and deregulated RhoA activity.

Tsc2 mutation or deletion has been used as a model for leiomyoma development since the characterization of the Eker rat. Indeed, mTOR signaling pathway up-regulation in human leiomyoma samples compared with normal control myometria is closely aligned with that in Eker rat leiomyomas compared with normal myometrium (Crabtree et al., 2009), which is consistent with the hypothesis that TSC2 mutation or deletion

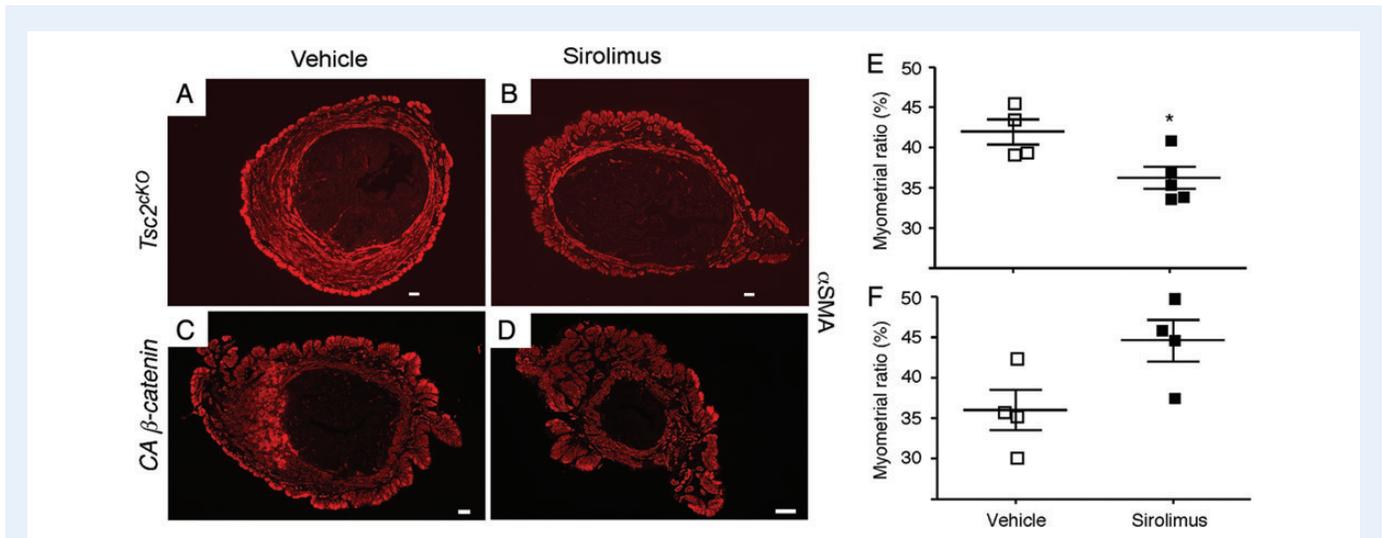


Figure 4 Sirolimus (rapamycin) treatment attenuates myometrial hyperplasia in *Tsc2^{ckO}* mice. Both *Tsc2^{ckO}* and CA β -catenin mice were treated for 6 weeks with either Sirolimus or vehicle control by oral gavage. Uteri were collected and myometrial hyperplasia was determined by α -SMA immunofluorescence. Representative photographs are shown in (A–D). Quantitative analysis of the ratio of myometrial area to the total uterine area in cross section was performed on uteri from $n \geq 4$ mice and plotted (E and F). The mean and SEM are shown as horizontal bars. * $P < 0.05$ by student's *t*-test. Bar = 100 μ m.

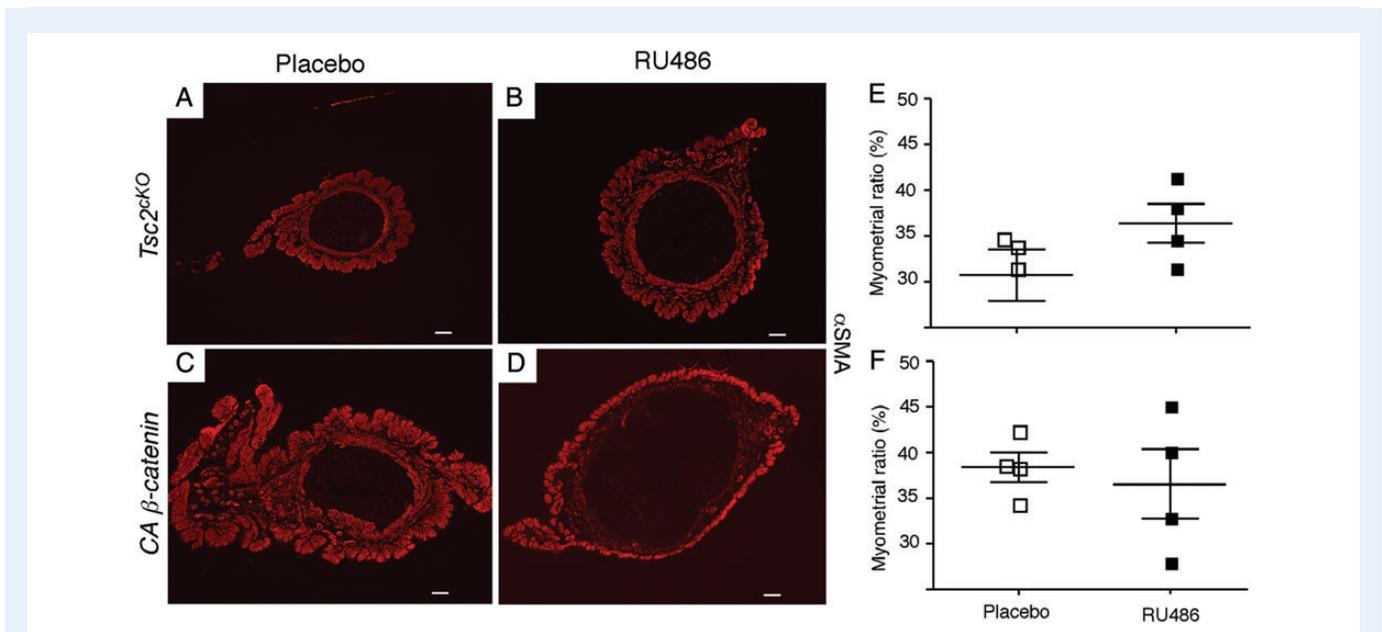


Figure 5 Mifepristone does not provide a significant therapeutic effect on myometrial hyperplasia in *Tsc2^{ckO}* mice. *Tsc2^{ckO}* and CA β -catenin mice ($n = 4$) were implanted with mifepristone or placebo timed-release pellets for 6 weeks. α -SMA immunofluorescence was used to assess myometrial hyperplasia in the uteri of the mice and representative photographs are shown (A–D). Quantitative analysis of the ratio of myometrial area to the total uterine area in cross section was performed on uteri and plotted (E and F). The mean and SEM are shown as horizontal bars. Bar = 100 μ m.

is associated with uterine leiomyomata. Additionally, a recent report describes a uterine leiomyoma-like phenotype in mice upon conditional deletion of TSC2 controlled by the progesterone receptor (*Pgr*) promoter-driven Cre (Prizant et al., 2013) whose expression is dramatically up-regulated in all post-natal uterine compartments when females begin cycling (Soyal et al., 2005). Notably however, the applicability of TSC2 loss to human leiomyoma development has been questioned by

a recent report suggesting that in human leiomyomata, TSC2 expression is actually increased compared with normal myometrium (Cui et al., 2011). Our inability to observe uterine leiomyomata by deletion of the *Tsc2* gene in the Müllerian duct mesenchyme-derived myometrium as reported here also suggests that loss of TSC2 alone is not sufficient for their development. Attempts to reconcile the lack of leiomyoma development we observed in the *Amhr2*-Cre mediated deletion of TSC2 with

the report that PR-Cre mediated deletion of TSC2 leads to their development in nearly 100% of mice by age 24 weeks (Prizant *et al.*, 2013) have provided two possible scenarios to account for the discrepancy in our respective models. A trivial answer would be the difference in Cre alleles used; whereas we used the *Amhr2-Cre*, they used the *Pgr-Cre*, which is more broadly expressed in uterine cells than the *Amhr2-Cre* and could trigger a more severe fibrosis phenotype akin to leiomyomas. A similar phenomenon was observed when the phenotypes of PR-Cre and *Amhr2-Cre* driven deletion of *Tsc1* were compared and found to be overlapping but distinct (Daikoku *et al.*, 2013). Another possible explanation is that haploinsufficiency or dosage sensitivity in PR expression can contribute to the phenotype. The *Pgr-Cre* allele is a knockin allele, which necessarily eliminates expression of PR from that allele and might lower PR expression levels. A related phenomenon was recently reported wherein the PR-Cre was used to delete the *Dicer* gene (Hawkins *et al.*, 2012). In that study, PR expression was lower in the mutant endometrium compared with controls. The authors suggested that the loss of *Dicer* could be affecting PR expression, which could also apply in PR-Cre-driven TSC2 loss. In either case, because progesterone is thought to play a stimulatory role in fibroid development and/or growth (Kim *et al.*, 2013), it is not clear how decreased expression of PR could provide a more permissive environment for tumorigenesis in PR-Cre mice. Surprisingly, however our studies with mifepristone, a PR antagonist, (Fig. 5) suggest that, although the study might be underpowered by using only four mice in each treatment group, there is either no effect or, at worst, a positive or promoting effect of mifepristone on hypertrophy in the *Tsc2*^{CKO} mice.

In summary, we have deleted *Tsc2* in mouse myometria and observed myometrial hypertrophy, which appeared to be reduced by rapamycin treatment and marginally induced by mifepristone therapies. In contrast, no myometrial phenotype was observed in *Tsc1*-deleted myometria (Supplementary data, Fig. S3). Future efforts at reconciling these results with those indicating that loss of TSC2 and/or TSC1 is a contributing factor to uterine leiomyoma development and progression should provide a better understanding of their etiology and pathophysiology.

Supplementary data

Supplementary data are available at <http://molehr.oxfordjournals.org/>.

Authors' roles

T.K.-T. was responsible for the conception of the study, acquisition of data, writing the draft of the manuscript and providing final approval. A.E.C. was responsible for acquisition of data, writing the draft of the manuscript and providing final approval. A.L.P., J.L.D., D.P. and A.K.S. were responsible for acquisition of data, revising the draft of the manuscript and providing final approval. J.M.T. was responsible for the conception and design of study, analysis of data, writing and revising the manuscript and providing final approval.

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Conflict of interest

None declared.

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